ROLE OF AMPA- NMDA-RECEPTORS AND BACK-PROPAGATING ACTION POTENTIALS IN SPIKE-TIMING DEPENDENT PLASTICITY

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ABSTRACT

The cellular mechanisms that mediate spike-timing dependent plasticity (STDP) are largely unknown. We investigated in vitro in CA1 pyramidal neurons the contribution of AMPA and NMDA components of Schaffer collateral (SC) EPSPs (EPSP\textsubscript{AMPA} and EPSP\textsubscript{NMDA}) and of the back-propagating action potential (BAP) to the LTP induced by a STDP protocol that consisted in pairing an EPSP and a BAP. Transient blockade of EPSP\textsubscript{AMPA} with CNQX during the STDP protocol prevented LTP. Contrastingly LTP was induced under transient inhibition of EPSP\textsubscript{AMPA} by combining SC stimulation, an imposed EPSP\textsubscript{AMPA}–like depolarization and BAP, or by coupling the EPSP\textsubscript{NMDA} evoked under sustained depolarization (~ -40mV) and BAP. In Mg\textsuperscript{2+}-free solution EPSP\textsubscript{NMDA} and BAP also produced LTP. Suppression of EPSP\textsubscript{NMDA} or BAP always prevented LTP. Thus, activation of NMDARs and BAP are required but not sufficient because AMPAR activation is also obligatory for STDP. However a transient depolarization of another origin that unblocks NMDARs and a BAP may also trigger LTP.

INTRODUCTION

Long-term potentiation (LTP) is an activity-dependent modification in synaptic efficacy that is thought to be the cellular substrate of the learning machinery in the brain (Bliss and Collingridge, 1993; Malenka and Bear, 2004; Nicoll and Malenka 1999). LTP may be induced by different protocols, but spike-timing dependent plasticity (STDP) appears to be closer to physiological conditions. STDP is a form of synaptic plasticity that obeys the associative ‘Hebbian’ learning rule (Hebb, 1949) and induces LTP by pairing at short delays a subthreshold excitatory postsynaptic potential (EPSP) with a back-propagating action potential (BAP) (Abbott and Nelson 2000; Bell et al. 1997; Bi and Poo 1998; Campanac and Debanne 2008; Debanne et al. 1996; Debanne et al. 1998; Markram et al. 1997; Nevian and Sakmann 2006; Sjostrum and Nelson 2002; Sjostrum et al. 2001).

We have shown that the waveform and amplitude of the Schaffer collateral (SC) EPSP determines the EPSP-BAP delay (i.e., the ‘temporal window’) required to induce and regulate the magnitude of the LTP (Fuenzalida et al. 2007). A likely interpretation of these results is that the initial depolarization provided by the activation of \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors (AMPARs) plays a key role in the regulation of the induction threshold and magnitude of LTP. The depolarization generated by the EPSP-BAP pairing induces a supralinear rise in spine Ca\textsuperscript{2+} concentration (Yuste and Denk 1995) mediated by the combined...
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activation of AMPARs, N-methyl-D-aspartate (NMDA) receptors (NMDARs) and voltage-gated
Ca\(^{2+}\) channels (VGCCs) (Golding et al. 2002; Kampa et al. 2006; Nevian and Sakmann 2006;
Schiller and Schiller 2001; Sjostrom and Nelson 2002; Yuste et al. 1999). However to what
extent the depolarization and Ca\(^{2+}\)- influx triggered by the AMPAR, NMDAR and VGCCs
activation and the BAP contribute in the induction of this LTP is largely unknown.

We investigated in hippocampal CA1 pyramidal neurons \textit{in vitro} the relative role of
EPSP\textsubscript{AMPA}, EPSP\textsubscript{NMDA} and BAP to the LTP induced by STDP that consisted in pairing at 10 ms a
subthreshold SC EPSP and BAP. Transient inhibition of EPSP\textsubscript{AMPA} with CNQX during the STDP
protocol prevented LTP. In contrast LTP was induced under transient blockade of EPSP\textsubscript{AMPA} by
pairing SC stimulation and a brief depolarization that precisely reproduced the EPSP\textsubscript{AMPA}
waveform (i.e., ‘simulated EPSP\textsubscript{AMPA}’) and BAP or by coupling a slight sustained depolarization
during the STDP protocol that unblocked NMDARs and triggered the EPSP\textsubscript{NMDA}. This LTP was
also induced in Mg\(^{2+}\)-free solution under transient blockade of EPSP\textsubscript{AMPA} during the STDP
protocol by pairing the EPSP\textsubscript{NMDA} and BAP. In contrast LTP was never induced under inhibition
of EPSP\textsubscript{NMDA} or in the absence of a BAP. We conclude that the rapid sequential activation of
NMDARs and BAP is required but not sufficient for LTP induction and that EPSP\textsubscript{AMPA} is
obligatory, implying an additional associative component in STDP.

MATERIALS AND METHODS

Procedures of animal care, surgery, and slice preparation were in accordance with the
guidelines laid down by the European Communities Council. The procedures will be described
briefly because they have been extensively detailed previously (Fuenzalida et al. 2007).

\textbf{Slice preparation.} Young Wistar rats (14 to 16 days old) were decapitated, and the brain
was removed and submerged in cold (~ 4°C) artificial cerebrospinal fluid (ACSF, in mm:
124.00 NaCl, 2.69 KCl, 1.25 KH\(_2\)PO\(_4\), 2.00 MgSO\(_4\), 26.00 NaHCO\(_3\), 2.00 CaCl\(_2\), 10.00 glucose).
In the Mg\(^{2+}\)-free solution MgSO\(_4\) was omitted. The pH of the ACSF was stabilized at 7.4 by
bubbling carbogen (95% O\(_2\), 5% CO\(_2\)). Transverse hippocampal slices (300-350 \textmu m) were cut
with a Vibratome (Pelco 3000, St Louis, MO, USA) and incubated in the ACSF (>1 hour, at
room temperature; 20-22\(^{\circ}\) C). Slices were transferred to a 2 ml chamber fixed to an upright
microscope stage (Olympus BX51WI, Tokyo, Japan) equipped with infrared differential
interference contrast (DIC) video microscopy and a 40x water immersion objective. Slices were
superfused with carbogen-bubbled ACSF (2 ml/minute) and maintained at room temperature. All
recordings were made under picrotoxin (PTX; 50 \textmu M), while 2-amino-5-phosphonopentanoic
acid (APV; 50 µM) and 7-nitro-2,3-dioxo-1,4-di hydroquinoxaline-6-carbonitrile (CNQX; 5 µM)
were added to the ACSF as needed.

**Electrophysiology.** Whole-cell recordings from soma of CA1 pyramidal cells were obtained with patch pipettes (4-8 MΩ) filled with an internal solution that contained in mM: 135 K-MeSO₄, 10 HEPES, 0.5 EGTA, 2 Na₂-ATP, 0.4 Na₃-GTP, buffered to pH 7.2 – 7.3 with KOH. Chemicals were purchased from Sigma-Aldrich Química (Madrid, Spain), Tocris (Biogen Científica, Madrid, Spain) and Alomone Labs. (Jerusalem, Israel). Recordings were performed both in the current- and voltage-clamp modes using an Axoclamp-2B amplifier (Molecular Devices Corporation, Chicago USA). Under voltage-clamp the membrane potential was fixed at ~ 65 mV, and in current-clamp conditions the membrane potential was set by injecting DC current as needed to ~ -65 mV; a value close to the average resting membrane potential of the cells analyzed (-67.9±7.8 mV; n=78). In some experiments the membrane potential was depolarized to ~ -40 mV by continuous current injection. Current-clamp recordings were rejected if in control conditions the membrane potential depolarized to values > -50 mV. In the voltage-clamp configuration the series resistance was compensated to ~ 70 % and neurons were accepted only when the seal resistance was > 1 GΩ and the series resistance (7-14 MΩ) did not change >10 % during the experiment. The liquid junction potential was measured (~ 6 mV) but was not corrected. Data were low-pass filtered at 3.0 kHz and sampled at rates between 6.0 and 10.0 kHz, through a Digidata 1322A (Axon Instruments, Inc.). The pClamp programs (Molecular Devices Corporation, Chicago, USA) were used to generate stimulus timing signals and transmembrane current pulses, and to record and analyze data. Stimulation with a bipolar extracellular electrode (60 µm diameter, tip separation ~ 100 µm) placed in the stratum radiatum ~ 100 µm from the soma of the recorded neuron evoked SC EPSCs at 0.3 Hz (**Fig. 1A**). A Grass S88 and SIU (Quincy, MA, USA) generated the simulation protocols under computer command.

**STDP protocols.** The CA1 pyramidal neuron was held at -65 mV under voltage-clamp and several of control excitatory postsynaptic current (EPSC) averages (n=20) evoked by repeated stimulation of SCs at 0.3 Hz were recorded to check response stability (**Fig. 1B**). The recording was then switched to the current-clamp mode, the membrane potential was adjusted to ~ -65 mV by injecting DC current as needed, and different STDP protocols were applied. The STDP protocols consisted in evoking SC EPSPs at 1.0 Hz with the same intensity as that used to evoke EPSCs under voltage–clamp, paired at a delay of 10 ms with a single BAP evoked by a transmembrane current pulse (3-5 ms, 0.5-2.0 nA) applied through the somatic recording electrode. Protocols were repeated 60 times at 1.0 Hz (Bi and Poo, 1998; Fuenzalida et al. 2007).
to test for the induction of LTP. Other STDP induction protocols applied under current-clamp
were performed under transient blockade of EPSP\textsubscript{AMPA} with a low concentration of CNQX (5
µM) applied during the STDP protocol and followed by a washout of the CNQX in control
solution. This low concentration of CNQX allowed a relatively rapid (~ 40 min) recovery of
EPSP\textsubscript{AMPA} under a washout in control solution. In some experiments the STDP protocol consisted
in combining SC stimulation followed at brief delay (~ 3 ms) by a ‘simulated EPSP\textsubscript{AMPA}’ that was
generated by injecting a current waveform through the somatic electrode. The current waveform
used to generate the ‘simulated EPSP\textsubscript{AMPA}’ in current-clamp recordings was a typical averaged
(n=20) SC EPSC obtained under voltage-clamp at ~ -65 mV and blockade of NMDARs with 50
µM APV. The ‘simulated EPSP\textsubscript{AMPA}’ was followed at 10 ms by a BAP (Fig. 2B, left). To allow
comparisons between experiments the amplitude of the real and ‘simulated EPSP\textsubscript{AMPA}’ was set to
similar values (4-6 mV) ~ 50 % under the threshold that could evoke spikes. The STDP induction
protocols consisted in (i) SC stimulation under transient blockade of EPSP\textsubscript{AMPA} plus the
‘simulated EPSP\textsubscript{AMPA}’; (ii) SC stimulation in Mg\textsuperscript{2+}-free solution under transient blockade of
EPSP\textsubscript{AMPA}; (iii) SC stimulation under transient blockade of EPSP\textsubscript{AMPA} plus sustained
depolarization to ~ -40 mV. In all cases recordings were then switched back to the voltage-clamp
mode and consecutive averages of EPSCs were obtained to determine the magnitude of the LTP
at -65 mV (Fig. 1B). In all conditions similar tests were performed in the absence of the BAP or
with NMDARs blocked by superfusion with 50 µM APV.

**Data Analysis:** Data were analyzed with the pClamp programs (Molecular Devices
Corporation, Chicago USA) and statistical evaluations with the Origin 7.0 (Originlab
Corporation, MA. USA). All voltage- and current-clamp responses were averaged (n=20) and the
magnitude of the change in EPSC peak amplitude was expressed as a proportion (%) of the
baseline control amplitude. Plots of the relative changes in EPSC peak amplitudes (% from
controls) versus time were constructed (Fig. 1C). Statistical analysis was performed using
Student’s two tail t-test and the differences were considered statistically significant at P < 0.05
level. Results are given as mean ± s.e.m (n=numbers of cells).

**RESULTS**

**Transient AMPAR blockade prevented LTP.** After attaining access to the intracellular
compartment we recorded under voltage-clamp several averaged control EPSCs (43.9±1.3 pA;
n=20) at -65 mV; see Methods) and < 20 min after (to avoid intracellular dialysis) switched to
current-clamp and applied the STDP protocol at ~ -65 mV (Fig. 1C). Upon return to the voltage-
clamp mode a robust long-lasting enhancement of the averaged EPSC peak amplitude that stabilized in ~ 20-30 min was recorded at -65 mV (191.7± 10.5%; P<0.05; n=10) (Fig. 1C, filled circles). Both blockade of NMDARs with 50 μM APV (n=5) (Fig. 1C, open circles) or exclusion of the BAP (n=5; not shown) prevented the induction of this LTP.

In related experiments (n=6) we recorded several averaged control EPSCs at -65 mV and rapidly (< 10 min after ‘braking in’) superfused 5 μM CNQX that inhibited both averaged EPSPs and EPSCs by ~ 90 % (Fig. 2A and B). In these conditions of pharmacological blockade of EPSPAMPA and voltage-dependent inhibition of NMDARs by extracellular Mg²⁺, the STDP protocol applied under current-clamp was unable to induce LTP as revealed after a washout (~ 40 min) in control solution to remove the CNQX-mediated inhibition of EPSCAMPA (Fig. 2A). The averaged control EPSCs recorded at -65 mV and after the EPSC peak amplitude stabilized in control solution were of similar amplitudes (Fig. 2A; P>0.05; n=6). In voltage-clamp conditions the control averaged EPSC was reduced by ~ 95 % when 5 μM CNQX was superfused (n=13), confirming a minor contribution of EPSPNMDA to the response at ~ -65 mV and the nearly total inhibition of AMPARs by CNQX (Fig. 2A and B, top right).

These results suggest that activation of NMDARs by stimulation of SCs and BAP are required but not sufficient to induce this LTP and that EPSPAMPA is obligatory for the induction of LTP by STDP. Therefore we investigated if EPSPAMPA could be substituted with an alternative depolarization.

**LTP was induced by a ‘simulated EPSP AMPA’ under AMPAR blockade.** We recorded under voltage-clamp control averaged EPSCs at -65 mV and blocked EPSPAMPA with 5 μM CNQX (as above). After switching to current-clamp we applied at ~ -65 mV a STDP protocol that consisted in pairing SC stimulation, a ‘simulated EPSPAMPA’ (see Methods) and a BAP delayed 10 ms. Following return to voltage-clamp at -65 mV a robust LTP (peak EPSCs amplitudes increased by 181.1 ± 9.7%; P<0.05; n=8) as revealed under voltage-clamp after a washout of CNQX (~ 40 min) in control solution (Fig. 2B). This LTP was also prevented by superfusion with 50 μM APV (98.1± 12.4; p<0.05; n=6; not shown) or by omitting the BAP (104.8± 4.9; p<0.05; n=6; not shown).

The above results taken together imply that EPSPAMPA may be substituted by a transient depolarization that unblocks NMDARs activated by the glutamate released through SC stimulation. Therefore the association of a transient membrane depolarization –either provided by AMPAR activation or by the ‘simulated EPSPAMPA’- combined with the activation of NMDARs; and the BAP are required and sufficient to induce this LTP.
Given that the depolarization provided by the ‘simulated EPSP\textsubscript{AMPA}’ induced LTP we tested if the pharmacologically isolated EPSP\textsubscript{NMDA} evoked under relief of the voltage-dependent Mg\textsuperscript{2+} blockade of NMDARs by an imposed depolarization could also induce LTP. Although a depolarization of a few millivolts should probably suffice because the depolarization provided the EPSP in control conditions was on the average 4.8±1.1 mV (n=10) we used larger depolarization to ~ -40 mV to guarantee that we were relieving the voltage-dependent Mg\textsuperscript{2+} blockade of NMDARs.

**LTP was induced at -40 mV under AMPAR blockade.** Several EPSCs were recorded under voltage-clamp at -65 mV and the averaged peak amplitudes were measured. EPSC\textsubscript{AMPA} was then blocked with 5 µM CNQX (as above), the recording was switched to current-clamp and the cell depolarized to ~ -40 mV after. Only cells that did not fire action potentials in these conditions were selected for this analysis. SC stimulation revealed a slow EPSP\textsubscript{NMDA} (~ 2 mV) that peaked at delays of ~ 30 ms (**Fig. 3A**). The STDP protocol applied in current-clamp conditions with the isolated EPSP\textsubscript{NMDA} at ~ -40 mV and BAP at a delay of 10 ms induced a robust LTP as revealed by the increased peak EPSC amplitude (by 151.1 ± 16.2 %, at ~ -65 mV; P<0.05; n=5) after a washout (~ 40 min) in control solution that eliminated the CNQX-mediated inhibition of AMPARs (**Fig. 3A**). The EPSP\textsubscript{NMDA} recorded at ~ -40 mV was caused by the relief of the Mg\textsuperscript{2+} blockade by the sustained depolarization to ~ -40 mV (Nowak et al., 1984) and was absent at ~ -65 mV (see above).

In these conditions of sustained depolarization we did not observe a significant reduction of the BAP peak amplitude that reached peak values of 44.2 ± 1.7mV in control conditions at ~ -65 mV and 43.2 ±1.5mV when depolarized to ~ -40 mV (n=5), probably indicating little Na\textsuperscript{+} inactivation close to the soma (~ 100 µm) where EPSP-EPSC was generated. In addition peak amplitudes were essentially identical at -65 mV when the BAP was preceded by the EPSP 45.1 ± 3.3mV (same cells). In addition we did not record any significant differences in the magnitude of the Ca\textsuperscript{2+} signals recorded from the apical dendrite 100 µm from the soma in the above conditions (see supplementary material 1). This LTP was also prevented by superfusion with 50 µM APV (98.9± 6.6; n p<0.05; n=6; not shown) or by omitting the BAP (94.5± 5.9; p<0.05; n=6; not shown).

Given that the depolarization induced by EPSP\textsubscript{AMPA} required to trigger this LTP could be substituted by an isolated EPSP\textsubscript{NMDA} evoked at -40 mV we analyzed if LTP could also be induced by the pharmacologically isolated EPSP\textsubscript{NMDA} evoked in Mg\textsuperscript{2+}-free solution followed by a BAP.
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LTP was induced in Mg\(^{2+}\)-free solution under AMPAR blockade. Slices were incubated in Mg\(^{2+}\)-free solution and several control averaged EPSC peak values (i.e. which in these conditions correspond to EPSC\(_{\text{AMPA}}\)) were measured under voltage-clamp at -65 mV. EPSC\(_{\text{AMPA}}\) was then blocked with 5 μM CNQX (as above) and after switching to current-clamp a STDP protocol was applied that consisted in pairing at ~ -65 mV the pharmacologically isolated EPSP\(_{\text{NMDA}}\) of ~ 2 mV with a BAP delayed 10 ms. After a washout (~ 40 min) in Mg\(^{2+}\)-free solution lacking CNQX there was a persistent enhancement of EPSC\(_{\text{AMPA}}\) (i.e., the EPSC peak amplitude increased by 136.6 ± 13.9%; P<0.05; n=8; Fig. 3B) as revealed under voltage-clamp at -65 mV. Therefore when NMDARs are isolated by CNQX a small NMDA-mediated membrane depolarization of ~ 2 mV may cause sufficient Ca\(^{2+}\)-influx to induce LTP. This LTP was also prevented by superfusion with 50 μM APV (103.0± 5.7; p<0.05; n=3; not shown) or by omitting the BAP (104.4± 6.0; p<0.05; n=3; not shown).

Obviously the contribution of the BAP to Ca\(^{2+}\)-influx in Mg\(^{2+}\)-free solution cannot be caused by the relief of the Mg\(^{2+}\) blockade by depolarization, because Mg\(^{2+}\) is absent in the extracellular solution. However, the depolarization and Ca\(^{2+}\)-influx supplied by the isolated EPSP\(_{\text{NMDA}}\) in Mg\(^{2+}\)-free solution could be sufficient to induce the LTP. To test any possible change in synaptic efficacy caused by Ca\(^{2+}\) influx through the unblocked NMDARs in the Mg\(^{2+}\)-free solution, the BAP was omitted throughout the experiment and control EPSC peak amplitudes (i.e., EPSC\(_{\text{AMPA}}\)) were recorded at -65 mV. EPSP\(_{\text{AMPA}}\) was then blocked with 5 μM CNQX, the recording was switched to current-clamp, and SCs were stimulated at 1 s\(^{-1}\) during 1 min while holding the cell at ~ -65 mV. Averaged EPSC\(_{\text{NMDA}}\) recorded under voltage-clamp in control solution and after a washout had essentially identical peak amplitudes at -65 mV (data no shown; P<0.05; n=4). Therefore, the isolated EPSP\(_{\text{NMDA}}\) could not induce the LTP in the absence of a BAP, most likely because it did not cause the required Ca\(^{2+}\)-influx. An interpretation of this negative result is that the BAP contributes by activating L-type VGCC that are a prime source of Ca\(^{2+}\)-influx into spines following dendritic depolarization by the BAP (Yuste et al., 1999). Nifedipine (20 µM) that specifically blocks L-type VGCC, prevented the LTP induced by the STDP protocol in Mg\(^{2+}\)-free solution without modifying control EPSCs (see supplementary material 2). Therefore the Ca\(^{2+}\)-influx required to induce the LTP was supplied by the combined activation of NMDARs by the released glutamate and of L-type channels activated by the BAP.

A key question that emerges from the above results is to establish which features of the membrane potential waveforms associated with the different STDP protocols are essential to induce LTP. Therefore, we compared the EPSP-BAP waveforms in control conditions and under blockade of AMPARs and NMDARs on current-clamp responses evoked by the STDP protocols.
Effects of CNQX and APV on current-clamp responses evoked by STDP protocols. The control current-clamp response shows a characteristic averaged EPSP-BAP sequence with a distinctive slow final spike after-depolarization (ADP) (Fig. 4A, black trace). Inhibition of NMDARs with 50 μM APV slightly reduced the amplitude of the averaged EPSP (to 92.5±7.35 % of control; P<0.05, n=6) and markedly reduced the spike ADP (to 41.9±7.1 % of control; P<0.05; same cells; single exponential fits were compared) (Fig. 4A, red trace). These results are consistent with a small contribution of NMDARs to the EPSP at the resting membrane potential and an important NMDA-mediated component in the spike ADP.

The EPSP virtually disappeared under superfusion with 5 μM CNQX (by ~ 95 %; n= 6), consistent with the inhibition of EPSP_{AMPA} and with the voltage-dependent blockade of EPSP_{NMDA} by extracellular Mg^{2+} at the resting membrane potential. In contrast the NMDA-mediated ADP component remained unchanged (91.5±8.2 % of control; p>0.05; n=6) implying that the ADP is not dependent on AMPAR activation (Fig. 4B, black trace). When 5 μM CNQX and 50 μM APV were added to the control solution there were no changes in the initial portion of the response (P>0.05; n=6), in harmony with the absence of EPSP_{AMPA} and the inhibition of EPSP_{NMDA}. In contrast there was a marked reduction of the NMDA-dependent contribution to the spike ADP (51.0 ± 11.2 % of control; p<0.05; same cells) (Fig. 4B, red trace).

We also compared the inhibition of AMPARs with 5 μM CNQX and of blockade of NMDARs with 50 μM APV on the membrane potential waveforms evoked by both the control and the ‘simulated EPSP_{AMPA}’ STDP protocols (n=5). The response under 5μM CNQX shows the characteristic ‘simulated EPSP_{AMPA}’-BAP sequence with the NMDA-mediated ADP component (Fig. 4C). The similarity between the control response (Fig. 4A, black trace) and that induced under inhibition of AMPARs with the added ‘simulated EPSP_{AMPA}’ (Fig. 4C) is consistent with the analogous LTP induction properties of both STDP protocols. The waveform of the averaged response evoked by the ‘simulated EPSP_{AMPA}’-STDP protocol under 50μM APV was essentially identical except for a decreased spike ADP (to 46.8 ± 6.1% of control; P<0.05; n=5) (Fig. 4C). Finally the waveform of the averaged response in Mg^{2+}-free solution under inhibition of AMPARs with 5μM CNQX was comparable to those in Fig. 4C except that the ADP amplitude was somewhat larger in the Mg^{2+}-free solution (19.5 ± 1.34 %; P<0.05; n=3; data not shown), in harmony with an NMDAR-mediated contribution to the spike ADP. Nifedipine did not modify the ADP suggesting that L-type VGCC do not contribute its generation.

The above results taken together confirm the: (i) insignificant contribution of NMDARs to the EPSP at the resting membrane potential; (ii) close similarity between the waveforms and
amplitudes real and ‘simulated EPSP\textsubscript{AMPA}’; and (iii) important NMDAR-mediated involvement in
the genesis of the ADP that is independent on the activation of AMPARs. However, except for
the spike ADP other differences in the BAP were not evident in somatic recordings.

DISCUSSION

We provide original evidence showing that in SC synapses the activation of NMDARs and
BAP are necessary but not sufficient to induce LTP and that EPSP\textsubscript{AMPA} is obligatory for STDP.
Nevertheless LTP may be induced in the absence of functional AMPARs if NMDARs are
unblocked by combining SC stimulation and a BAP added with a ‘simulated EPSP\textsubscript{AMPA}', or a
small imposed depolarization (-40mV); or else in Mg\textsuperscript{2+}-free solution. We also prove that
depolarization, activation of NMDARs and BAP are obligatory for the induction of LTP in all the
above conditions. However, it has been proposed that the ADP may contribute to the LTD
induced by STDP (Karmarkar et al., 2002).

LTP requires Ca\textsuperscript{2+} influx into spines through NMDARs freed by the EPSP\textsubscript{AMPA}–mediated
depolarization that although small (a few mV) is able to relieve the block by extracellular Mg\textsuperscript{2+}
(Abbott and Nelson 2000; Bliss and Collingridge 1993; Malinow and, Malenka, 2002; Yuste et
al. 1999). With STDP the rapid sequential EPSP-BAP activation depolarizes spines causing the
supralinear Ca\textsuperscript{2+} influx both through NMDARs and VGCC (Fuenzalida et al., 2007; Kampa et al.
2006; Nevin and Sakmann, 2006; Sjostrom and Nelson 2002; Yuste and Denk 1995; Yuste et al.
1999; present Results). Therefore in CA1 pyramidal cells VGCC trigger dendritic Ca\textsuperscript{2+} spikes
that are required for LTP induction with STDP protocols (present results; Campanac and
Debanne 2008; Golding et al. 2002; Yuste and Denk 1995; Yuste et al. 1999). Therefore similar
mechanisms where the small AMPA-mediated depolarization followed by the BAP induce a
substantial increase in the spine Ca\textsuperscript{2+} concentration may be at work in the natural condition
because evidence has been provided indicating that Ca\textsuperscript{2+} spikes that span over a section of the
dendrite are required to induce LTP (Kampa et al. 2006).

It has been argued that that with STDP a single BAP does not provide the necessary
depolarization to trigger dendritic Ca\textsuperscript{2+} spikes for LTP induction at SC synapses, and that a burst
of two or more BAPs are obligatory, or else that larger prolonged spikes evoked under K\textsuperscript{+}
channel blockade are required (e.g., Nishiyama et al. 2000; Pike et al., 1999; Wittenberg et al.,
2006). The discrepancy with our results is difficult to identify, although differences in the size of
the EPSP, that has to be large to induce LTP (Debanne et al. 1996; Debanne et al. 1998;
Spine depolarization and STDP

Fuenzalida et al. 2007; Sjostrom and Nelson 2002; Sjostrom et al. 2001), or in the position of the stimulating electrode, that has to be near the soma where the attenuation of BAPs along the apical dendrite is minimal may contribute (e.g., Campanac and Debanne 2008; Letzkus et al. 2006; Johnston et al. 2003; see below). An added divergence is that the CA1 area was isolated by removal of CA3 in those reports, a situation that could modify conduction and synaptic transmission in SCs. However, other reports agree with the present findings because they show that single pre-postsynaptic pairing invariantly induces LTP in cultured hippocampal neurons (Bi and Poo 1998) slice cultures (Debanne et al. 1996; Debanne et al. 1998) and in acute slices (Fuenzalida et al. 2007). In addition a robust LTP is induced in mature animals with single postsynaptic spikes when GABA A receptors are blocked with PTX (Campanac and Debanne 2008).

The spread of BAP over the apical dendrites is regulated by several factors, where the transient K+-mediated Ia (Hoffman et al., 1997), the hyperpolarization-activated Na+ and K+-mediated Ih (Fan et al. 2005; Sjostrom and Nelson 2002; Tsay et al. 2007) and the slow Ca2+-activated K+ mediated sI AHP play important roles by constraining dendritic depolarization, the generation of Ca2+ spikes and the propagation of synaptic signals. Interestingly, both Ia and Ih may be reduced by depolarization, the former because it is inactivated and the latter since it is deactivated by the decreased Ca2+-influx. Repeated activity-induced Ca2+ influx may facilitate the sI AHP within minutes (Borde et al. 1995) and prevent STDP by reducing SC EPSPs (Fuenzalida et al., 2007), most likely mainly by shunting the NMDA component (Fernandez de Sevilla et al. 2002). In addition classical LTP induction protocols may cause a long-term enhancement of the sAHP that prevents non-potentiated synaptic inputs to exhibit LTP (Le Ray et al. 2004). The Ia, Ih and sAHP have preferred spatial distributions over the apical dendritic tree of CA1 pyramidal neurons, thus the induction of LTP could depend on the location of activated synapses over the apical dendrites, as has been shown in cortical pyramidal neurons where STDP follows different learning rules along the apical dendrite (Letzkus et al. 2006). Rules for STDP may also depend on system specializations, as occurs in the electrosensory lobe of mormyrid fish, where the EPSP-BAP association generates depression instead of LTP (Bell et al. 1997).

In control conditions EPSPs depolarize activated spines and subsequently spread over a stretch of dendrite. The extension of the dendritic depolarization depends on the number and position of the SC synapses activated and on the functional state of the apical dendrite (Johnston et al. 2003; Fernandez de Sevilla et al. 2007) a process in which dendritic Ca2+ spikes triggered by the BAP play a crucial role (Kampa et al. 2006; Nevian and Sakmann, 2006; Yuste and Denk 1995; Yuste et al. 1999). In contrast the depolarization induced by the combined SC stimulation-
simulated EPSP\textsubscript{AMPA}’ spreads from the soma to the dendrites. Surprisingly both STDP protocols induce essentially identical LTPs probably because the spine Ca\textsuperscript{2+}-influx caused by those protocols are of analogous magnitude. This conclusion is consistent with the close similarity between the depolarizing response waveforms evoked by both protocols.

SC synapses may be either ‘functional’, that contain both AMPARs and NMDARs and conduct at the resting membrane potential, or ‘silent’ that only express NMDARs and conduct when NMDARs are unblocked by depolarization (Cabezas and Buno, 2006; Fernandez de Sevilla et al. 2002; Isaac et al. 1995). LTP is thought to result from the functional conversion of ‘silent synapses’ by the insertion of AMPARs into spines triggered by depolarization and Ca\textsuperscript{2+} influx (Isaac et al. 1995). With the control STDP protocol the spine depolarization generated by EPSP\textsubscript{AMPA} in functional synapses spreads over the adjacent dendrite to nearby activated silent synapses unblocking NMDARs that with the BAP provide the required supralinear Ca\textsuperscript{2+} influx to induce the functional conversion. The depolarization evoked by the SC stimulation-‘simulated EPSP\textsubscript{AMPA}’–BAP sequence spreads from the soma and depolarizes spines of ‘silent synapses’ to trigger the functional conversion. A similar interpretation may hold both for the LTPs induced by adding a slight (~ 40mV) sustained depolarization and in Mg\textsuperscript{2+}-free solution.

Although it has been argued that the high spine neck resistance filters electric signals and causes electrical and chemical isolation of the spine, present results suggest that the electrical isolation of spines may not be rigid and may in special conditions permit the spread of electrical signals from and to the adjacent dendrite (Yuste et al. 1999; Majewska et al. 2000; Bloodgood and Sabatini, 2005; Grunditz et al. 2008).

We conclude that with STDP protocols NMDARs activation and BAP are both required but not sufficient for LTP induction and that the initial subthreshold depolarization supplied by AMPAR activation is obligatory for the induction and regulation of the LTP. Therefore the essential requirement of EPSP\textsubscript{AMPA} implies an additional associative component that could have important consequences for the functional conversion of silent synapses. We demonstrate that SC stimulation coupled with a brief depolarization from another source and a BAP also induces LTP, most likely by causing the necessary supralinear Ca\textsuperscript{2+} influx in the absence of AMPAR activation. We also provide evidence indicating that the Ca\textsuperscript{2+} influx is supplied by the combined activation of NMDARs and L-type VGCCs. It may be argued that in the absence EPSP\textsubscript{AMPA} other mechanisms might provide the required depolarization for STDP if associated with BAPs. Depolarization may either be mediated may by intrinsic membrane properties (Garcia-Munoz et al. 1993) or by transmitters and neuromodulators (Bonansco and Buno 2003; Dodd and Kelly 1981; Haas and Konnerth, 1983; Lin et al. 2003; Minke 2006; Schiller and Schiller 2001). Finally
we supply indirect evidence suggesting a permissive bidirectional electrical pathway between dendrite and spine.
REFERENCES


FIGURE LEGENDS

Figure 1. LTP induced by STDP and effects of blocking NMDARs. A, Diagram of Schaffer collateral (SC) stimulation and somatic patch recording of CA1 pyramidal cell. B, top. Experimental protocol showing Control EPSC average (n=20 as in other cases) evoked by SC stimulation under voltage-clamp. EPSP-BAP response evoked by STDP protocol (10 ms delay, repeated at 1/sec 60 times) in current-clamp conditions; the lower record shows the stimulation protocol corresponding to responses shown above. EPSC average recorded under voltage-clamp 30 min later showing the typical response amplification of LTP. C, bottom. Time course of the changes in EPSC peak amplitude (% of controls) induced by the STDP protocol (open arrow, as in all other figures) in control solution (filled circles; n=10) and under blockade of NMDARs (50μM APV; open circles; n=5). B, top. Averaged EPSCs recorded before (1) and 30 min after the STDP protocol (2); the superimposed 1 and 2 EPSCs are also shown (1+2). All records obtained at ~ -65 mV.

Figure 2. Effects of CNQX and of ‘simulated EPSP AMPA’. A, left. Representative average record evoked under current-clamp during STDP protocol with blockade of AMPARs (5μM CNQX); SC stimulation (Stim) and transmembrane current Pulse are shown below. A, bottom right. Time course of the changes in EPSC peak amplitude induced by superfusion with 5 μM CNQX (horizontal bar) followed by the STDP protocol and a prolonged washout (n=6). A, top right. Averaged control EPSCs recorded before (1) and immediately after the STDP protocol (2) and after a prolonged washout (3). Note the blockade of the EPSP by CNQX and the recovery to control amplitudes after a washout. B, Left. Same as A, left but STDP protocol with added ‘simulated EPSP AMPA’ (EPSPsim). B, bottom. Same as A, bottom right but STDP protocol with the added ‘simulated EPSP AMPA’ and after a prolonged washout (n=8). B, top right. Averaged control EPSCs recorded before (1), immediately after the STDP protocol (2) and after a prolonged washout (3). Note the block of the EPSC by CNQX and the LTP revealed following a washout. All records obtained at ~ -65 mV.

Figure 3. LTP induced under transient AMPAR blockade by STDP at -40 mV and in Mg2+-free solution. A, left. Representative average record evoked under current-clamp at -40 mV during STDP protocol with blockade of AMPARs (5μM CNQX); SC stimulation (Stim) and transmembrane current Pulse are shown below. Note the NMDA-mediated EPSP. A, bottom right. Time course of EPSC peak amplitude (i.e., AMPA response component) changes induced at ~ -65 mV by the STDP protocol under 5 μM CNQX in Mg2+-free solution and after a
prolonged washout (>40 min) showing recovery from AMPAR blockade and LTP (n=5). A, top. Averaged control EPSCs recorded before (1), during (2) and 30 min after the STDP protocol and a washout (3) in control solution. B, left. Same as A, left, but at -65 mV and in Mg²⁺-free solution. B, bottom right. Same as A, bottom right but at -65 mV and in Mg²⁺-free solution (n=8). B, top right. Averaged control EPSCs recorded before (1), immediately after the STDP protocol (2) and after a prolonged washout (3).

**Figure 4. Current-clamp response average waveforms in the different experimental conditions.** A, Representative average current-clamp records showing response evoked by STDP protocol (10 ms) recorded at ~ -65 mV in control solution and under 50 μM APV. The arrow indicates the NMDA-mediated ADP. B, same as A, but with 5 μM CNQX and 5 μM CNQX plus 50 μM APV. C, Responses evoked by the STDP protocol and added ‘simulated EPSP_{AMPA}’ in control solution and with 5 μM CNQX.
SUPPLEMENTARY MATERIAL

1. **Membrane depolarization does not modify the BAP peak amplitude recorded at the soma nor the Ca$^{2+}$ signal obtained at the apical dendrite.**

   **A, left,** Superimposed BAPs (upper) evoked by depolarizing pulses of identical intensity at -60 and -40 mV (thin and thick traces, respectively). The corresponding Ca$^{2+}$ signals (lower) recorded at the apical dendrite 100 µm from the soma. Note the similarity of the BAPs in both conditions and the different ADPs; the corresponding Ca$^{2+}$ signals were also essentially identical. **A, center,** same as **A, left,** but in one experiment the BAP was preceded by a SC EPSC (thick record). Note the absence of the similarity of the responses that follow in both conditions (upper); the Ca$^{2+}$ signals (lower) were also essentially identical. **A, right,** summary data showing the absence of effect of membrane depolarization in the above conditions (n=5 in each case).

2. **The induction of LTP is inhibited by nifedipine in Mg$^{2+}$-free solution.**

   **B, upper, left,** EPSP-BAP sequence evoked under blockade of L-type channels in Mg$^{2+}$-free solution. Note that the EPSP and the ADP are present. **B, lower, left,** EPSCs peak amplitudes were similar under nifedipine in Mg$^{2+}$-free solution before (1) and following (2) the STDP protocol (30 min) indicating the absence of LTP. **B, right,** plot showing average peak amplitudes of EPSCs as a function of time, under blockade of L-type channels with nifedipine (20 µM) and in Mg$^{2+}$-free solution (n=4). Note the absence of effect of the STDP protocol.
Stimulation

CA1 Pyramidal cell

SC Recording

20 ms

10 mV

20 pA

EPSC EPSP-BAP

Control LTP STDP

20 ms

20 pA

50 ms

% EPSP - BAP

1 + 2

20 pA

50 ms

% EPSP - BAP

1 + 2

20 pA

50 ms

Figure 1
Figure 2
Figure 3

A

EPSC Amplitude (%)

EPSP\textsubscript{NMDA-BAP} -40mV

Control solution

Time (minutes)

B

EPSP Amplitude (%)

EPSP\textsubscript{NMDA-BAP} -65mV

Mg\textsuperscript{2+}-free solution

Time (minutes)
Figure 4